Attorney Docket No. 03715.0148-00000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Etienne-Emile BAULIEU et al.	Group Art Unit: 1616
Serial No.: 10/542,495	Examiner: Mei Ping Chui
Filed: January 18, 2006	Confirmation No.: 7023
For: USE OF 3-METHOXY-PREGNENOLONE IN THE PRODUCTION OF A MEDICAMENT FOR TREATING NEURODEGENERATIVE DISEASES	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Etienne-Emile BAULIEU, do hereby make the following declaration:

1. I am a citizen of France.

2. My short curriculum vitae, is attached as **Exhibit A**. Briefly, I obtained an MD from Faculté de Médecine and a Ph.D. from Faculté des Sciences, both of Paris. I am currently Director of Research Team 3 at Inserm UMR788; CEO of MAPREG, which is an R&D Biopharmaceutical Company dedicated to developing and marketing innovative products for the treatment of nervous system disorders; and President of the Institut Professeur Baulieu, which aims to understand issues that are detrimental to health in people of age and thus to decrease dependability problems of the elderly.

Among other awards, I was awarded laureate of a Lasker Clinical Medical Research Award in 1989. In addition, I am a foreign associate member of the United

States National Academy of Sciences since 1990, and member of the French Académie des Sciences and of the Académie Nationale de Médecine.

3. I am one of the inventors of the above-referenced application, U.S. Patent Application 10/542,495, filed January 18, 2006 and am familiar with its content, including the amended claims attached hereto as **Exhibit B**.

4. I have read an Office Action dated May 8, 2009, a copy of which is attached as **Exhibit C**.

5. I have read and understand the disclosure of U.S. Patent No. 6,245,757 by Chopp *et al.* (*Chopp*), filed October 1, 1998, as well as U.S. Patent Application Publication No. 2002/0072509 by Stein *et al.* (*Stein*), filed October 9, 2001.

6. I understand that the pending claims have been rejected under 35 U.S.C. § 103(a) as allegedly obvious over both *Chopp* and *Stein* in view of the Chemical Book.

7. *Stein*, while listing numerous molecules under the heading "progestins," only tested progesterone and two of its downstream metabolites, epipregnanolone and allopregnanolone, for efficacy in a model of traumatic brain injury. Only allopregnanolone achieved a statistically significant effect in a post-trauma animal learning model. *See* Figure 2 and paragraph [0082] of *Stein*. Allopregnanolone is a known GABA receptor agonist. *See Stein* at paragraph [0006]; *see also* **Exhibit D**.

8. *Chopp* describes experiments using progesterone in an animal model of ischemic brain injury. *See* Examples in *Chopp*. *Chopp* recognizes that once progesterone crosses the blood-brain barrier, it is metabolized into more active forms, such as 3α, 5α THP (allopregnanolone, *see, e.g.*, **Exhibit D**). *Chopp* further opines that

progesterone's alleged neuroprotective effect following ischemia is due, at least in part, to its GABA receptor agonist activity. *See Chopp* at paragraph bridging columns 11 and 12.

9. I believe that person having ordinary skill in the art in 2003, considering *Chopp* and *Stein* would, *a priori*, believe that any neuroprotective effects of "progestins" would likely be mediated by progesterone or its downstream metabolites, such as allopregnanolone, acting as GABA receptor agonists.

10. Although *Chopp* and *Stein* list pregnenolone and pregnenolone methyl ether (which I understand the Examiner considers to be the same as to 3-methoxy-PREG) as "progestins," it would be clear to one having ordinary skill in the art in 2003 that this classification is erroneous. A skilled artisan would have understood, even before considering *Chopp* and *Stein's* experimental results, that progestin (also called a progestogen or progestagen) is by definition a compound that has biological activity substantially similar to progesterone, *e.g.*, a compound that binds the progesterone receptor (PR) and affects various downstream biological activities. Accordingly, pregnenolone (PREG) would not have been considered a progestin—it is not a downstream metabolite of progesterone, but only a precursor of progesterone and, in contrast to progesterone or allopregnanolone, it is a GABA receptor *antagnoist*. *See* **Exhibit E**. 3-methoxy-PREG is also not a progestin—and is, in fact, an antagonist of the progesterone receptor. *See* **Exhibit F**. Furthermore, due to the presence of the 3-methoxy group, 3-methoxy-PREG (as well as other molecules of formula I in claim 1) cannot be converted into progesterone or its downstream active metabolites, such as

allopregnanolone, *in vivo*. *See also* specification at page 3, lines 22-26 and page 4, lines 6-11.

11. The experiments described in **Exhibit F** were performed by me or under my supervision. The results of these experiments further demonstrate that 3-methoxy-PREG would not have been considered a progestin by a person having ordinary skill in the art since: **1)** it does not exhibit progesterone receptor agonist activity, **2)** it acts as a progesterone receptor antagonist, and **3)** it does not bind other steroid hormone receptors.

12. Figure 1 of **Exhibit F** shows the results of a cell-based luciferase reporter assay for progesterone receptor agonist activity. These results illustrate that, unlike progesterone, 3-methoxy-PREG does not induce transactivation of a progesterone receptor luciferase reporter, even at the highest concentrations tested. Thus, 3-methoxy-PREG does not have progesterone receptor agonist activity.

13. Figure 2 of **Exhibit F** shows the results of a cell-based luciferase reporter assay for progesterone receptor antagonist activity. These results illustrate that 3-methoxy-PREG inhibits progesterone receptor activation by progesterone in a dose-dependent manner at concentrations greater than approximately 10^{-6} M. Thus, 3-methoxy-PREG would have been considered a weak progesterone receptor antagonist by one having ordinary skill in the art.

14. Table 2 of **Exhibit F** summarizes the results of cell-based receptor-binding assays testing the ability of 3-methoxy-PREG to displace natural cognate ligands of several steroid receptors. These results demonstrate that 3-methoxy-PREG does not

bind any of the steroid hormone receptors tested (including aldosterone, Androgen (testosterone) AR, Estrogen ER (α or β), and glucocorticoid) to any significant degree.

14. As described in Example 3 of the application, 3-methoxy-PREG stimulates neurite outgrowth in cultures of PC12 cells in the presence of NGF by stimulating MAP2-dependent microtubule polymerization (see Example 2 of the application).

15. **Exhibit G** is a peer-reviewed study, which confirms that progesterone does not stimulate microtubule polymerization. *See* Figure 1B of **Exhibit G**; *see also* Example 2 of the application.

16. Accordingly, not only would the skilled artisan not have considered 3-methoxy-PREG a progestin, but in addition, the canonical progestin, progesterone, does not share 3-methoxy-PREG's microtubule polymerization-stimulating activity.

17. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Nov 4, 2009

By: [signature]
Etienne-Emile BAULIEU

Exhibit A of Declaration under 37 C.F.R § 1.132
of
Etienne-Emile BAULIEU

EXHIBIT A - SHORT CURRICULUM VITAE OF Pr BAULIEU

NAME Baulieu, Etienne-Emile	POSITION TITLE Professor at Collège de France Adjunct Professor at Scripps
COMMONS USER NAME	

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Faculté de Médecine, Paris	M.D.	1955	Endocrinology
Faculté des Sciences, Paris	Ph. D.	1963	Biochemistry

Positions and honors

Positions and employment :

1951-1955 Resident, Paris Hospitals.
1955-1957 Chef de Clinique, Paris University.
1958 Associate Professor of Biochemistry, Paris University.
1970-1993 Professor of Biochemistry – Paris-Sud University.
1993 Professor of Human Reproduction – Collège de France.
1963 INSERM – Scientific director.
1964-1997 Roussel Uclaf – Consultant.
2000 MAPREG Compagny, Founder and President

Public functions :

Editorial Boards of several French and International Journals.
Scientific Committees : Fondation pour la Recherche Médicale Française (Former President, 1973-1974) and.INSERM (Former President, 1975-1979).
Société Française d'Endocrinologie (Past-President, 1978).
Karolinska Symposia on Reproductive Endocrinology (member of Organizing Committee).
Special program in Human Reproduction of WHO (former member of the Scientific Committee).
National Academy of Sciences of the USA, Foreign Associate (1989).
Honorary Member, The American Physiological Society (1993)
Associated Professor at the Karolinska Institutet (Stockholm, 1994).
Academia Europea, Member (1995)
Comité Consultatif National d'Ethique (CCNE), Member (1996-2004)
Société de Secours des Amis des Sciences, Président (1999).
Académie Nationale de Médecine, Member (2002).
Institut de la Longévité et du Vieillissement (Founder, 2002)
Académie des Sciences, Member (1982), President (2003-2004).
Comité d'Initiative et de Proposition pour la recherche scientifique (CIP): co-President.
Scripps Institute, Adjunct Professor (2006)

Honors :

Lauréat Assistance Publique (Médaille de l'Internat), Académie Nationale de Médecine, (Prix Fondation Dreyfous, 1956, Prix Specia, 1964), and Académie des Sciences (1960).
Reichstein Award of International Society of Endocrinology (1972).
Roussel Prize (with E. Jensen, 1976)
Gregory Pincus Memorial Award (with E. Jensen, 1978).
First European Medalist of the Society of Endocrinology (G.B., 1985).
A. and E. Wippman Scientific Research Award, Planned Parenthood of America (1989).
Albert and Mary Lasker Clinical Research Award (1989).
Claude Bernard Lecture, Royal Society (1990).
Premio Minerva, Roma (1990)

Christopher Columbus Discovery Award in Biomedical Research (Genova and NIH, 1992).
National Award, the American Association for Clinical Chemistry (1992).
Daniel Perey Lecture (Canada, 1993).
Joseph Bolivar DeLee Humanitarian Award (Chicago, 1994).
Grand Prix de la Fondation pour la Recherche Médicale (1994).
Ken Myer Medal (Melbourne, Australia, 2000).
Academy of Humanism, Laureate (2002).
Distinguished Science Achievement Award(Oakland University, 2002)
Sawyer Distinguished Award (Los Angeles, UCLA, 2003)
Grand Officier de la Légion d'Honneur (2003).

Doctor Honoris Causa, Université de Gand (1991).
Honorary degree, Tufts University (1991).
Doctor Honoris Causa, Karolinska Institutet (1994).
Honorary Degree, Worcester Foundation for Experimental Biology, USA (1994).

Main books:

Hormones (with P. Kelly): Hermann (Paris) and Chapman & Hall (New York) publishers, 1990.

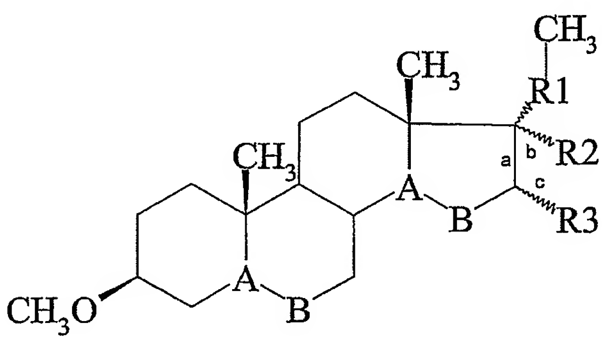
Generation Pilule, Pub. Odile Jacob, 1990.

The Abortion Pill, Simon & Schuster publisher (New York), 1991.

Contraception : contrainte ou liberté? (with F. Héritier and H. Léridon), Pub. Odile Jacob, 1999.
Neurosteroids: A New Regulatory Function in the Nervous System, (with P. Robel P. and M. Schumacher (Humana Press, coll. Contemporary Endocrinology), 1999.

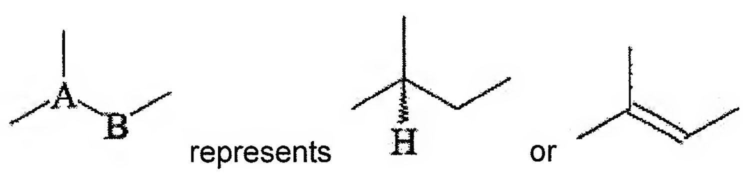
Exhibit B of Declaration under 37 C.F.R § 1.132
of
Etienne-Emile BAULIEU

1. (Currently amended) A method for treating an acute or chronic lesion or a degenerative disease of the nervous system ~~by stimulating the polymerization and/or the stabilization of microtubules~~ in a patient, comprising ~~the administration to said~~ <u>administering to the</u> patient ~~of an effective quantity of a drug~~ <u>a composition</u> comprising 3β-methoxy-pregna-5-ene-20-one (3-methoxy-PREG) or a molecule derived from pregnenolone that contains a 3-methoxy function and is incapable of being converted into a metabolite or ester sulfate of pregnenolone, wherein said molecule derived from pregnenolone is of formula I:



(I)

in which:

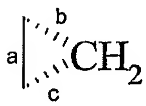
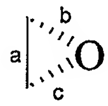


R1 = –CO–; –CH(OH)– or –CH(O-COCH$_3$)–

R2 = H or CHCl$_2$,

R3 = H or CH$_3$, or

R2 and R3 together form a ring:

 or 

wherein the composition is administered to the patient in an amount effective to stimulate polymerization and/or stabilization of microtubules in the patient.

2. (Previously Presented) The method according to claim 1, wherein said disease or lesion is selected from the group comprising Alzheimer's disease, Parkinson's disease, age-induced memory loss, memory loss induced by the taking of substances, a traumatic lesion, a cerebral lesion, a lesion of the spinal cord, in particular medullary compression, ischemia, pain, notably neuritic pain, nerve degeneration, and multiple sclerosis.

3. (Currently amended) The method according to claim 1, wherein said ~~drug~~ composition also comprises an excipient that makes it possible to formulate the molecule derived from pregnenolone to cross the blood-brain barrier.

4. (Currently amended) The method according to claim 1, wherein said ~~drug~~ composition is administered by injection.

5. (Currently amended) The method according to claim 1, wherein said ~~drug~~ composition is administered orally.

6. (Previously Presented) The method according to claim 1, wherein said molecule of formula I is 3-methoxy-PREG.

7. (Withdrawn) The method according to claim 1, wherein said molecule of formula I is 3β-methoxy-pregna-5-ene-20-one-17α-dichloromethyl.

8. (Currently amended) The method according to claim 1, wherein said ~~drug~~ composition comprises a quantity of 3-methoxy-PREG or of said molecule of formula I ranging between 50 and 2500 mg.

9-10. (Cancelled)

11. (Withdrawn) An in vitro method for increasing the stabilization and/or inducing the polymerization of the microtubules in a cell, comprising the step of exposing the aforementioned cell to the presence of 3-methoxy-pregnenolone at a concentration of approximately 0.5 to 50 µmol.

12. (Withdrawn) An in vitro method for increasing neuritic sprouting in a cell, comprising the step of exposing the aforementioned cell to the presence of 3-methoxy-pregnenolone at a concentration of approximately 0.5 to 50 µmol.

13. (New) The method of claim 1, wherein the acute or chronic lesion or a degenerative disease of the nervous system is not ischemia.

Exhibit C of Declaration under 37 C.F.R § 1.132
of
Etienne-Emile BAULIEU

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/542,495	01/18/2006	Etienne-Emile Baulieu	03715.0148	7023

22852 7590 05/08/2009
FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER LLP
901 NEW YORK AVENUE, NW
WASHINGTON, DC 20001-4413

EXAMINER	
CHUI, MEI PING	

ART UNIT	PAPER NUMBER
1616	

MAIL DATE	DELIVERY MODE
05/08/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/542,495	BAULIEU ET AL.	
	Examiner	**Art Unit**	
	MEI-PING CHUI	1616	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *22 July 2008*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-12* is/are pending in the application.

4a) Of the above claim(s) *7,11 and 12* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-6 and 8-10* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *15 July 2005* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *n/a*.

4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

5) ☐ Notice of Informal Patent Application

6) ☐ Other: _____.

DETAILED ACTION

Status of Action

Receipt of Amendments/Remarks filed on 07/22/2008 is acknowledged. Claims 1-12 are pending in this application. Claims 1-6, 8-10 have been amended in the response filed on 07/22/2008.

Upon further consideration, Applicants' amendments necessitated the new grounds of rejection presented in this Office Action. Accordingly, this action is made **FINAL**.

Priority

Acknowledgment is made of Applicants' claim for foreign priority based on an application filed in France on 01/17/2003. However, it is noted that Applicants have not filed a certified copy of the English translation of the foreign application No. 03/00507 as required by 35 U.S.C. 119(b).

Status of Claims

Accordingly, claims **1-6, 8-10** are presented for examination on the merits for patentability as they read upon the elected subject matter and claims **7, 11-12** directed to non-elected inventions are withdrawn.

Rejections and/or objections not reiterated from the previous Office Action are hereby withdrawn. The following rejections and/or objections are either reiterated or newly applied. They constitute the complete set of rejections and/or objections presently being applied to the instant application.

Response to Arguments

Applicants' arguments filed on 07/22/2008, with respect to claims 1-6, 8-10, have been considered but are moot in view of the new ground(s) of rejection necessitated by Applicants' amendments.

New Grounds of Claim Rejection

Claim Rejections - 35 USC § 112 second paragraph

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 1-2, 4-6 and 8 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. All dependent claims are included in this rejection.

Claim 1 recites a treatment method "comprising the administration to a patient of an effective amount of a drug comprising 3β-methoxy-pregna-5-ene-20-one or a molecule derived from pregnenolone that contains a 3-methoxy function...", which is indefinite because the general accepted plain meaning of "a drug" is defined as **"a substance"** used as a medicament or in the preparation of medicament, or a substance intended for use as a component of a medicine (see Merriam-Webster Online Dictionary-Definition for Drug). It is unclear whether Applicants intend to claim the drug itself (contains only 3β-methoxy-pregna-5-ene-20-one or a molecule derived from pregnenolone that contains a 3-methoxy function) is administered to a patient or Applicants intend to claim the drug, which may also contain administration vehicle, i.e.

carrier, is administered to a patient. Therefore, one of ordinary skill in the art would not be reasonably apprised of the scope of the invention, and thus rendering the claim indefinite.

Claims 2, 4 and 8 are also rejected because they depend from claim1, and thus incorporate its limitation.

Claim Rejection - 35 U.S.C. § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102(b) that form the basis for the rejections under this section made in this Office action:

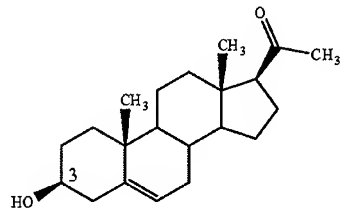
A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

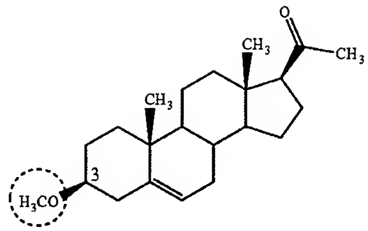
Claim 9 is rejected under 35 U.S.C. 102(b) as being anticipated by The Merck Index (Twelfth Edition, 1996: page 1328, compound 7915), as evidenced by Chemical Book (retrieved via www.chemicalbook.com for the structure of pregnenolone methyl ether).

Instant claim 9 is directed to a drug consisting of 3-methoxy-pregnenolone (or refers as 3-methoxy-PREG in instant claim).

The Merck Index (1996) discloses the structure of the compound pregnenolone (page 1328: compound No. 7915 and see below) and discloses the physical property of its methyl ether derivative: pregnenolone methyl ether (page 1328: compound 7915, last two lines and see structure below):

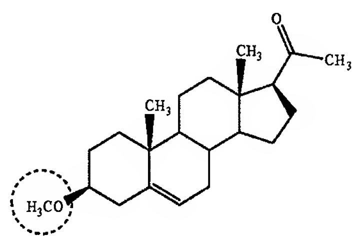


pregnenolone (parent compound)



pregnenolone methyl ether

Since the methylation of the parent compound pregnenolone can only occur at the C3-hydroxyl position to form the methyl ether derivative, the disclosure of methyl ether of pregnenolone will necessarily be the structure as set forth above (the structure at the right), as evidenced by Chemical Book (retrieved via www.chemicalbook.com), which discloses the structure of pregnenolone methyl ether (see Chemical Book printout and structure below):



pregnenolone methyl ether

Therefore, the disclosure of Merck Index anticipates instant claim 9.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

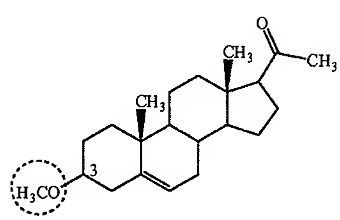
The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

(1) Claims 1-6, 8 and 10 are rejected under 35 U.S.C. 103(a) as being unpatentable over Chopp et al. (U. S. Patent No. 6,245,757), as evidenced by Chemical Book (retrieved via www.chemicalbook.com for the structure of pregnenolone methyl ether).

Applicants Claim

Applicants claim a method of treating an acute or chronic lesion, or a degenerative disease of the nervous system, comprising the administration to a patient of a drug comprising 3β-methoxy-pregna-5-ene-20-one or a molecule derived from pregnenolone of the formula I (see below structure):



3-methoxy-pregnenolone

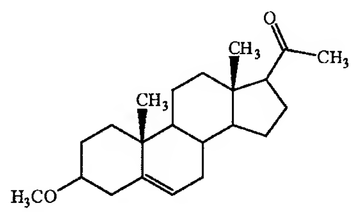
Determination of the scope and content of the prior art

(MPEP 2141.01)

Chopp et al. teach a method for the treatment of ischemic damage, i.e. damage due to stroke, comprising administering to a mammal afflicted with ischemic cell damage an effective amount of a pharmaceutical composition comprising progestin and a

pharmaceutically acceptable delivery vehicle (see Abstract and column 2, lines 28-45). Chopp et al. also teach that the method functions by the ability of the progestin to reduce the damage caused by ischemia, i.e. brain damage caused by cerebral ischemia, and the significant neurological functions improvement, as well as the enhancement of the ability of the brain to recognize after damage, be enhancing its intrinsic ability to compensate for injury (column 2, lines 38-45). As a result, the method provides whereby ischemic tissue, including tissue of the central nervous system or muscle tissue, can be treated so as to improve tissue survival and to hasten general bodily recovery (column 4, lines 22-26).

Chopp et al. then teach that the useful progestins for the treatment include pregnenolone methyl ether (column 5, lines 4-5 and structure below):



pregnenolone methyl ether

Chopp et al. further teach that the progestin can be formulated as pharmaceutical formulations and administered to a mammal, i.e. human patient, in a variety of unit dosage forms, i.e. injection, adapted to the chosen route of administration, i.e. orally or parenterally includes intravenous route (column 5, lines 55-60 ad column 6, line 43). For oral administration, the progestin can combine with or more pharmaceutical excipients, so that the progestin is formulated to pass through the blood-brain barrier and enters the central nervous system at widespread sites and can effectively reduce infarct size following acute, focal ischemia, i.e. middle cerebral artery occlusion, when given before and after the onset of ischemia (column 6, lines 1-2; column 12, Example 2: lines 9-11; column 3, lines 63-67; column 4, line 1 and column 5, line 4).

In addition, Chopp et al. teach that each unit dosage form comprises the active progestin in amounts from 5-1000 mg (column 7, lines 14-17). Therefore, it meets the limitation for the claimed 3-methoxy-PREG is present in amounts "ranging from 50 to 2500 mg" as claimed in claim 8.

With respect to the recitation of the types of disease, i.e. an acute lesion, memory loss induced by a traumatic lesion, a cerebral lesion, ischemia, as claimed in claim 1 and claim 2, Chopp et al. teach that the treatment method utilizes the progestin for reducing ischemic damage due to stroke or myocardial infarction (see Abstract), wherein the treatable ischemia can be resulted from brain damage caused by cerebral ischemia (column 2, lines 38-41 and column 4, lines 9-12) or can be trauma resulting from ischemic insult (column 4, lines 13-20). It is also known that stroke is a type of acute ischemia and is commonly referred as brain ischemia or acute ischemic stroke. Therefore, the teaching of Chopp et al. meets the limitation of the recitation "an acute lesion" in claim 1 and the recitation "a traumatic lesion", "a cerebral lesion" and "ischemia", as claimed in claim 2.

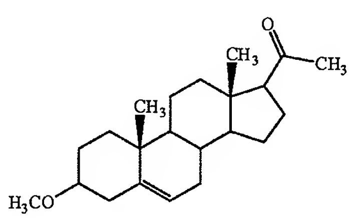
With respect to the recitation of "wherein the drug also comprises an excipient that makes it possible to formulate...." in claim 3 is an optional claim language (see MPEP 2106 (II)). Further, Applicants broadly claim "an excipient" without any structural limitation. Therefore, the examiner takes the position that any excipient taught in the prior art reads on the recitation of claim 3 for the reason set forth above, since the prior art excipient and the claimed excipient are not structurally distinguish. In order to be limiting, the intended use must create a structural difference between the claimed

composition and the prior art composition. In the instant case, the intended use does not create a structural difference, thus the intended use is not limiting.

Ascertainment of the difference between the prior art and the claims (MPEP 2141.02)

Chopp et al. teach a method of treating ischemic damage, i.e. damage due to stroke, comprising administering to a mammal an effective amount of a pharmaceutical composition comprising progestin, i.e. pregnenolone methyl ether, and a pharmaceutically acceptable delivery vehicle.

Chopp et al. do not particularly exemplify the use of pregnenolone methyl ether in the examples. However, Chopp et al. suggest that pregnenolone methyl ether can be used as one of the useful progestin for treating ischemic damage (see structure below and the pregnenolone methyl ether structure retrieved from Chemical Book via www.chemicalbook.com, as evidenced):



pregnenolone methyl ether

Finding of prima facie obviousness Rational and Motivation (MPEP 2142-2143)

It would have been obvious to a person of ordinary skilled in the art at the time the invention was made to follow the guidance of Chopp et al. to arrive at the instant invention.

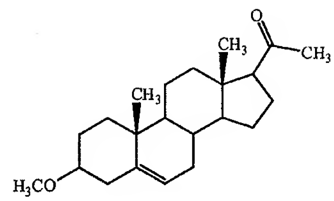
One of ordinary skill would have been motivated to do this because the prior art, namely Chopp et al., has already taught an effective method of treating ischemic damage, i.e. acute cerebral ischemia or brain ischemia, by administering to a human patient an effective amount of progestin in combination with an excipient. One of ordinary skill also would have been motivated to try the useful progestin, as suggested by Chopp et al., and then choose a desirable progestin and uses in the same method for treating ischemia, i.e. cerebral ischemia, as taught by Chopp et al.

From the teaching of the references, one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention, as a whole, would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

(2) Claims 1-6, 8 and 10 are rejected under 35 U.S.C. 103(a) as being unpatentable over Stein et al. (U. S. Patent No. 2002/0072509), as evidenced by Chemical Book (retrieved via www.chemicalbook.com for the structure of pregnenolone methyl ether).

Applicants Claim

Applicants claim a method of treating an acute or chronic lesion, or a degenerative disease of the nervous system, comprising the administration to a patient of a drug comprising 3β-methoxy-pregna-5-ene-20-one or a molecule derived from pregnenolone of the formula I (see below structure):



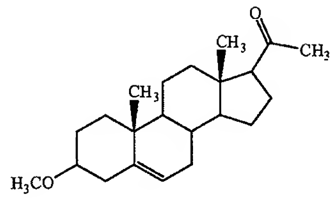
3-methoxy-pregnenolone

Determination of the scope and content of the prior art

(MPEP 2141.01)

Stein et al. teach a method and a composition for the treatment of neuro-degeneration following a traumatic injury to the central nervous system by reducing, or eliminating, neuronal cell death, edema, ischemia, and enhancing tissue viability, such that the treatment can enhance survival, proliferation, or/and neurite outgrowth of the neurons that either prevents or retards neuro-degeneration, i.e. a progressive loss of neurons in the central nervous system (page 2: [0016], lines 1-8). Stein et al. teach that the physiological events lead to the neuro-degeneration of the CNS tissues following a traumatic CNS injury, i.e. cerebral edema, increase in the immune and inflammatory response, demyelinization (page 2: [0017], lines 1-6).

Stein et al. also teach that the neuro-protective method is achieved by the administration of a therapeutically effective composition comprising a progestin, or a progestin metabolite, to a patient, i.e. human, wherein the useful progestin, i.e. pregnenolone methyl ether, can be used the method (page 2: [0018], lines 26-27 and 40, and the structure below), as evidenced by Chemical Book for the structure of pregnenolone methyl ether structure (retrieved via www.chemicalbook.com):



3-methoxy-pregnenolone

Stein et al. further teach that the composition may further comprise a pharmaceutically acceptable carrier vehicle, and the composition can be prepared into a pharmaceutically useful composition suitable for all forms of dose administration, i.e. injection and oral. Stein et al. teach that due to the traumatic CNS injury, the blood brain barrier may be more permeable for allowing the active compound to enter the cerebral spinal fluid (page 5: [0036-0037] and [0039-0041]).

With respect to the suitable amount of progestin in a dose, Stein et al. teach that such amount can be varied from about 1 μg to about 50 mg per kg of average body weight for the administration to a patient (which corresponds to about 14 mg to about 3500 mg per 70 kg of average body weight) (page 5: [0036-0037] and [0039-0041]).

Ascertainment of the difference between the prior art and the claims

(MPEP 2141.02)

Stein et al. teach a method utilizing a composition, which comprises an effective amount of progestin, i.e. pregnenolone methyl ether, and a pharmaceutically acceptable carrier vehicle for the treatment of neuro-degeneration following a traumatic injury to the central nervous system.

Stein et al. do not particularly exemplify the use of pregnenolone methyl ether in the examples. However, Stein et al. suggest that <u>pregnenolone methyl ether</u> can be used

as one of the useful progestin for protecting neuro-degeneration following a traumatic injury and enhance survival, proliferation, or/and neurite outgrowth of the neurons that either prevents or retards neuro-degeneration.

Finding of prima facie obviousness Rational and Motivation (MPEP 2142-2143)

It would have been obvious to a person of ordinary skilled in the art at the time the invention was made to follow the guidance of Stein et al. to arrive at the instant invention.

One of ordinary skill would have been motivated to do this because the prior art, namely Stein et al., has already taught an effective method of treating neuro-degeneration following a traumatic injury to the central nervous system by reducing, or eliminating, neuronal cell death, edema, ischemia, and enhances survival, proliferation, or/and neurite outgrowth of the neurons that either prevents or retards neuro-degeneration, i.e. a progressive loss of neurons in the central nervous system, by administering to a human patient an effective amount of progestin in combination with a pharmaceutically acceptable carrier vehicle. One of ordinary skill also would have been motivated to try the useful progestin, as suggested by Stein et al., and then choose a desirable progestin for use in the same method of preventing progressive loss of neurons in the central nervous system, as taught by Stein et al.

From the teaching of the references, one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention, as a whole, would have been *prima facie* obvious to one of ordinary skill in

the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Conclusion

No claims are allowed.

Applicant's amendment necessitated the new grounds of rejection presented in this Office Action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Contact Information

Any inquiry concerning this communication from the Examiner should direct to Helen Mei-Ping Chui whose telephone number is 571-272-9078. The examiner can normally be reached on Monday-Thursday (7:30 am – 5:00 pm). If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor Johann Richter can be

reached on 571-272-0646. The fax phone number for the organization where the application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either PRIVATE PAIR or PUBLIC PAIR. Status information for unpublished applications is available through PRIVATE PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the PRIVATE PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/H. C./

Examiner, Art Unit 1616

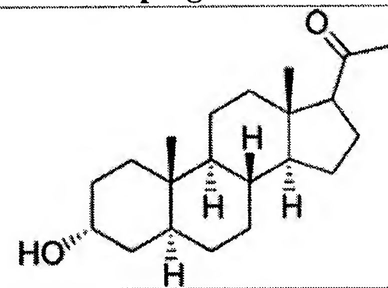
/Johann R. Richter/

Supervisory Patent Examiner, Art Unit 1616

Exhibit D of Declaration under 37 C.F.R § 1.132
of
Etienne-Emile BAULIEU

Allopregnanolone

From Wikipedia, the free encyclopedia

Allopregnanolone	
	
IUPAC name	1-(3-Hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-17-yl)ethanone
Other names	3α,5α-Tetrahydroprogesterone
Identifiers	
CAS number	516-54-1
PubChem	262961
SMILES	`[H][C@]34[C@]2([H])CC[C@@]1([H])C[C@H](O)CC[C@@](C)1[C@]([H])2CC[C@@](C)3C(C(C)=O)CC4`
Properties	
Molecular formula	$C_{21}H_{34}O_2$
Molar mass	318.49 g/mol
Except where noted otherwise, data are given for materials in their standard state (at 25 °C, 100 kPa)	
Infobox references	

Allopregnanolone, also known as **3α,5α-tetrahydroprogesterone** or **THP**, is an important neurosteroid in the human brain. It is a metabolite of progesterone and a barbiturate-like modulator of central gamma-aminobutyric acid (GABA) receptors that modify a range of behaviors, including the stress response.

The 5β epimer of this compound is known as pregnanolone, and has very similar properties to allopregnanolone. Both compounds are found endogenously and have similar hypnotic and anxiolytic effects.

References

* Herd, MB,; Belelli D, Lambert JJ. (2007). *Neurosteroid modulation of synaptic and extrasynaptic GABA(A) receptors*. Pharmacol. Ther. 116(1):20-34. doi:10.1016/j.pharmthera.2007.03.007 (http://dx.doi.org/10.1016%2Fj.pharmthera.2007.03.007) .

Retrieved from "http://en.wikipedia.org/wiki/Allopregnanolone"
Categories: Neurosteroids

Exhibit E of Declaration under 37 C.F.R § 1.132
of
Etienne-Emile BAULIEU

1. Ciba Found Symp. 1990;153:83-97; discussion 97-106.

Steroid regulation of the GABAA receptor: ligand binding, chloride transport and behaviour.

Majewska MD.

Addiction Research Center, National Institute on Drug Abuse, Baltimore, MD 21224.

Certain endogenous steroids are modulators of GABAA receptors. Tetrahydroprogesterone (THP, 5 alpha-pregnan-3 alpha-ol-20-one) and tetrahydrodeoxy-corticosterone (THDOC, 5 alpha-pregnane-3 alpha, 21-diol-20-one) behave as allosteric agonists of GABAA receptors whereas pregnenolone sulphate acts as an antagonist. THP and THDOC modulate ligand binding to GABAA receptors like barbiturates; they potentiate binding of the GABAA receptor agonist muscimol and the benzodiazepine flunitrazepam and they allosterically inhibit binding of the convulsant t-butylbicyclophosphorothionate. THP and THDOC also stimulate chloride uptake and currents in synaptoneurosomes and neurons. Pregnenolone sulphate acts principally as an allosteric GABAA receptor antagonist; it competitively inhibits binding of [35S] TBPS and blocks GABA agonist-activated Cl- uptake and currents in synaptoneurosomes and neurons. In behavioural experiments the GABA-agonistic steroid THDOC shows anxiolytic actions whereas the GABA-antagonistic steroid pregnenolone sulphate antagonizes barbiturate-induced hypnosis. Changes in physiological levels of GABAergic steroids may alter GABAA receptor function, influencing neuronal excitability and CNS arousal. For example, pregnancy and the puerperium are associated with alterations in GABAA receptor binding which might be attributable to steroid actions.

PMID: 1963401 [PubMed - indexed for MEDLINE]

Exhibit F of Declaration under 37 C.F.R § 1.132
of
Etienne-Emile BAULIEU

The main experimental setting used in the first two experiments below is the following: HEK293T cells were transiently transfected, using calcium phosphate precipitation technology, with expression vectors pSG5hPR (which permits expression of human progesterone receptor(PR)), pFC31-luc (contains the luciferase gene under the control of the MMTV promoter, which is in turn activated by binding of a progestin to progesterone receptor) and pcbetagal (which permits expression of betagalactosidase), and cultured during 24 hours with increasing amounts of various compositions.

I. Test of progesterone receptor agonist activity

Transfected cells were cultured with increasing amounts of progesterone or 3-methoxy-pregnenolone.

With this setting, a compound with progesterone receptor agonist activity permits a transactivation activity resulting in the expression of luciferase (since the binding of a progestin to PR results in activation of the MMTV promoter, which directs the expression of luciferase). In contrast, a compound without progesterone receptor agonist activity does not permit a transactivation activity and luciferase is not expressed (since PR is not activated and thus does not activate the MMTV promoter).

Results are shown in Figure 1.

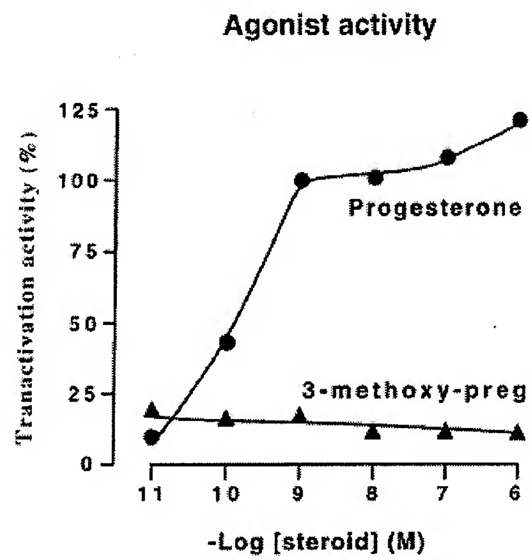


Figure 1. Test of progesterone receptor agonist activity

II Test of progesterone receptor antagonist activity

Transfected cells were cultured with progesterone (1 nM) and increasing amounts of RU486 (a progesterone receptor antagonist) or 3-methoxy-pregnenolone.

With this setting, a compound with progesterone receptor antagonist activity competes with progesterone for the occupation of progesterone receptor and results in a progressive loss of transactivation activity when the amount of this compound is increased compared to progesterone.

Results are shown in Figure 2.

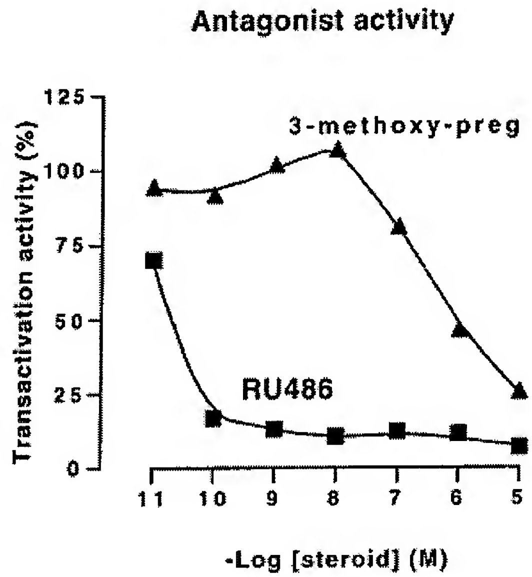


Figure 2. Test of progesterone receptor antagonist activity

III. Test of 3-methoxy-pregnenolone Steroid Hormone Receptor Binding Activity

For all receptors tested, radioligand binding assays were used. Briefly, cells expressing the tested receptor were incubated with their radiolabelled cognate ligand and the same non-radiolabelled ligand, in the presence or absence of various concentrations of non-radiolabelled 3-methoxy-PREG (MAP4343), the highest concentration tested for 3-methoxy-PREG (MAP4343) being 10 μM.

The particular cells and incubation conditions used are detailed in Table 1, below for each tested receptor:

Tested receptor	Cells expressing receptor	Radio-labelled ligand	Non-radio-labelled ligand	Vehicle	Incubation time / temperature	Incubation buffer
Aldosterone	Wistar Rat kidney	4.5 nM [.H] D-Aldosterone	3 µM D-Aldosterone	1% DMSO	60 minutes / 25.°C	100 mM Tris-HCl, pH 7.4, 3 mM CaCl.
Androgen (Testosterone) AR	Rat recombinant E. coli	1.5 nM [.H] Mibolerone	10 µM Mibolerone	1% DMSO	4 hours / 4.C	50 mM Tris-HCl, pH 7.4, 0.8 M NaCl, 10% Glycerol, 2 mM Dithiothreitol, 0.1% BSA, 2% EtOH
Estrogen ERα or ERβ	Human recombinant insect Sf9 cells	0.5 nM [.H] Estradiol	1 µM Diethylstilbestrol	1% DMSO	2 hours / 25.C	10 mM Tris-HCl, pH 7.4, 0.1% BSA, 10% Glycerol, 1 mM DTT
Glucocorticoid	Human HeLa S3 cells	3 nM [.H] Dexamethasone	10 µM Dexamethasone	1% DMSO	2 hours / 25.C	10 mM HEPES/RPMI-1640, pH 7.2

The amount of radiolabelled cognate ligand bound to cells expressing the tested receptor was then compared in the absence of 3-methoxy-PREG (MAP4343) or in the presence of 3-methoxy-PREG (MAP4343) and a percentage of inhibition was calculated as follows:

If the amount of radiolabelled ligand is decreased in the presence of 3-methoxy-PREG, then:

% inhibition = (amount or bound radiolabelled ligand in the presence of 3-methoxy-PREG) / (amount or bound radiolabelled ligand in the presence of 3-methoxy-PREG) * 100.

If the amount of radiolabelled ligand is increased in the presence of 3-methoxy-PREG), then:

% inhibition = - (amount or bound radiolabelled ligand in the presence of 3-methoxy-PREG) / (amount or bound radiolabelled ligand in the presence of 3-methoxy-PREG) * 100.

A significant binding at the tested concentration is present if % inhibition is over 50%.

Results are shown in Table 2, below:

Receptor tested	**Species**	**3-methoxy-PREG concentration tested**	**% inhibition**
Aldosterone	Rat	10 μM	25
Androgen (Testosterone) AR	Rat	10 μM	18
Estrogen ERα	Human	10 μM	-8
Estrogen ERβ	Human	10 μM	16
Glucocorticoid	Human	10 μM	21

* a significant binding is found if % inhibition is superior to 50%

Exhibit G of Declaration under 37 C.F.R § 1.132
of
Etienne-Emile BAULIEU

Microtubule-associated protein 2 (MAP2) is a neurosteroid receptor

Virginie Fontaine-Lenoir*, Béatrice Chambraud†, Arlette Fellous*, Sébastien David*, Yann Duchossoy*, Etienne-Emile Baulieu*†‡, and Paul Robel*†‡

*MAPREG Company, Centre Hospitalier Universitaire de Bicêtre, Bâtiment Paul Langevin, 78, Rue du Général Leclerc, 94275 Le Kremlin Bicêtre Cedex, France; and †Institut National de la Santé et de la Recherche Médicale U788, Stéroides et Système Nerveux, 80 Rue du Général Leclerc, 94276 Le Kremlin-Bicêtre Cedex, France

Contributed by Etienne-Emile Baulieu, January 10, 2006

The neurosteroid pregnenolone (PREG) and its chemically synthesized analog 3β-methoxypregnenolone (MePREG) bind to microtubule-associated protein 2 (MAP2) and stimulate the polymerization of microtubules. PREG, MePREG, and progesterone (PROG; the physiological immediate metabolite of PREG) significantly enhance neurite outgrowth of nerve growth factor-pretreated PC12 cells. However, PROG, although it binds to MAP2, does not increase the immunostaining of MAP2, contrary to PREG and MePREG. Nocodazole, a microtubule-disrupting agent, induces a major retraction of neurites in control cultures, but pretreatment with PREG/MePREG is protective. Decreasing MAP2 expression by RNA interference does not modify PROG action, but it prevents the stimulatory effects of PREG and MePREG on neurite extension, showing that MAP2 is their specific receptor.

RNA interference | PC12 | neurites | pregnenolone | neuroprotection

The brain is a target organ for steroid hormones (see ref. 1 for review). Intracellular receptors that are involved in the regulation of specific gene transcription have been identified in neuroendocrine structures, and they account for the many molecular events that are involved in steroid hormone action. However, the characterization of pregnenolone (PREG) in the rat brain (at higher concentrations than in blood) and its persistence after removal of steroidogenic endocrine glands (adrenals and gonads) (2) led to the discovery of a steroid biosynthetic pathway in the nervous system (3). PREG, which retains the carbon skeleton of cholesterol, is the precursor of steroid hormones, but does not bind to any nuclear receptor of steroid hormones (4). PREG sulfate was found to allosterically modulate several neurotransmitter receptors (namely GABA type A, NMDA-type glutamate, and sigma 1 receptors) (5); however, the neuromodulatory effects of neurosteroids could hardly account for their tentatively described neurotrophic and neuroprotective activities. Therefore, it was disappointing not to find receptors proper for the most abundant neurosteroid, PREG.

Rat brain cytosol contains a PREG-binding protein, which is identified as the microtubule associated protein 2 (MAP2), and PREG stimulates MAP2-driven microtubule assembly (6). Electron microscopic observation of PREG-induced microtubules shows their normal conformation, in contrast to microtubules that are assembled in presence of Taxol. Progesterone (PROG), which also binds to MAP2, does not stimulate microtubule polymerization, but counteracts the effect of PREG (as do competitive antagonists).

Microtubules are major structural components of the neuronal cytoskeleton, and they have an essential role in the elaboration of axons and dendrites (7). MAP2 has an important role in neuronal morphogenesis. The specific suppression of MAP2 synthesis prevents the neuronal differentiation of embryonic carcinoma cells exposed to retinoic acid, as shown by the absence of neurites (8).

The PC12 clonal rat pheochromocytoma cell line of neural crest origin, when exposed in culture to nerve growth factor (NGF), ceases to divide and undergoes neuronal differentiation characterized by the elongation of neuritic processes (9). MAP2 is present in PC12 cells during differentiation (10, 11), and there is a direct correlation between the increase of neurite length and the rate of microtubule assembly (12). Here, we examined the neurotrophic and neuroprotective activities of PREG, its 3-methylether derivative 3β-methoxypregnenolone (MePREG) (patent no. WO2004/067010) and PROG. Then, we used RNA interference (RNAi) to definitely demonstrate the involvement of MAP2 in the action of PREG and MePREG, but not of PROG, thus accounting for an uncharacterized mechanism of steroid action.

Results

Kinetics of Microtubule Polymerization *in Vitro*. The experiments were carried out with either purified rat brain microtubules (1 mg/ml protein) or PC12 cell cytosol (4 mg/ml protein).

Control microtubules that were purified from rat brain started to polymerize after a time lag of ≈90 s, with a steep initial slope during the first 5 min, followed by a smooth increase up to a variation of OD (ΔOD) of 0.153 after 15 min (Fig. 1*a*). Rat brain microtubules that were incubated with either PREG or MePREG (40 μM) polymerized faster and reached a ΔOD that was almost the same with PREG and MePREG (0.249 and 0.258, respectively) after 15 min; these ΔODs were 63% or 68% larger than the control level, respectively. Moreover, both steroids increased the initial rate of microtubule polymerization. The effect of PROG was not assayed because it is known not to stimulate the polymerization of microtubules prepared from mammalian brain (6). The purpose of this experiment was to compare the effects of PREG and MePREG.

The speed of formation and the amount of microtubules prepared from PC12 cell cytosol, even after prolonged incubation for 30 min, were definitely smaller than those of depolymerized rat brain microtubules and did not reach a plateau level (Fig. 1*b*). MePREG was more active than PREG (after 30 min, ΔODs were 84% and 67% larger than the control level, respectively). PROG did not stimulate microtubule polymerization.

Effects of Steroids on NGF-Induced PC12 Neurite Outgrowth. Observation of α-tubulin immunostaining suggested that PC12 cells, cultured in presence of NGF (10 ng/ml) and treated with either PREG or MePREG or PROG (30 μM) for 6 days, had longer neurites than those in control cultures (Fig. 2*a*).

Conflict of interest statement: No conflicts declared.

Abbreviations: NGF, nerve growth factor; ΔOD, variation of OD; MAP, microtubule-associated protein; MePREG, 3β-methoxypregnenolone; PREG, pregnenolone; PROG, progesterone; RNAi, RNA interference; siRNA, small interfering RNA.

‡To whom correspondence may be addressed. E-mail: baulieu@kb.inserm.fr or robel@kb.inserm.fr.

NEUROSCIENCE

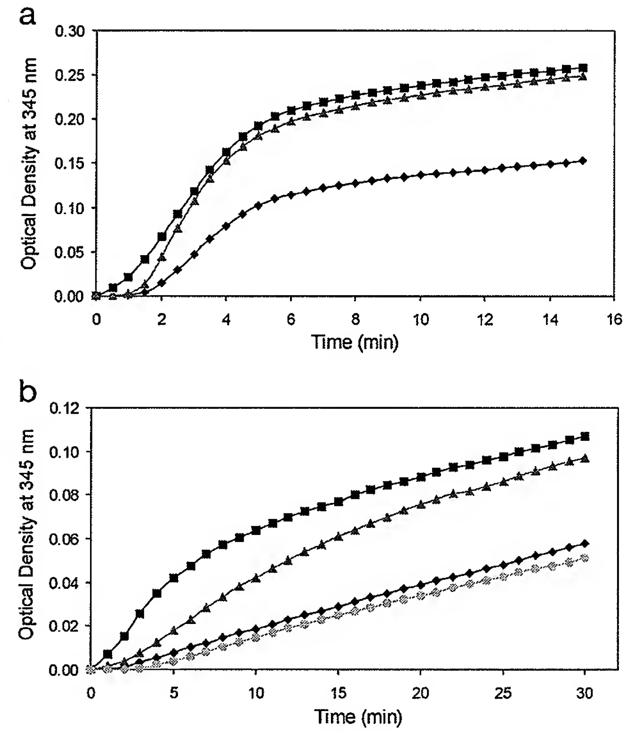


Fig. 1. Effects of steroids on tubulin polymerization *in vitro*. Assembly of microtubules was monitored at 345 nm at 37°C and recorded every 30 s. The quantity of microtubule formed is proportional to the increase of OD (ΔOD). (*a*) Microtubule proteins purified from rat brain cytosol (1 mg/ml) were incubated either without steroid (black diamonds) or with 40 μM PREG (gray triangles) or 40 μM MePREG (black squares) for 15 min. Results represent means for six independent observations. (*b*) PC12 cell cytosol (4 mg/ml) was incubated without steroid (black diamonds) or with PREG (gray triangles), MePREG (black squares), or PROG (gray circles) (40 μM) for 30 min. Results represent means for three independent observations. PREG and MePREG stimulated microtubule polymerization in both conditions, whereas PROG was inactive.

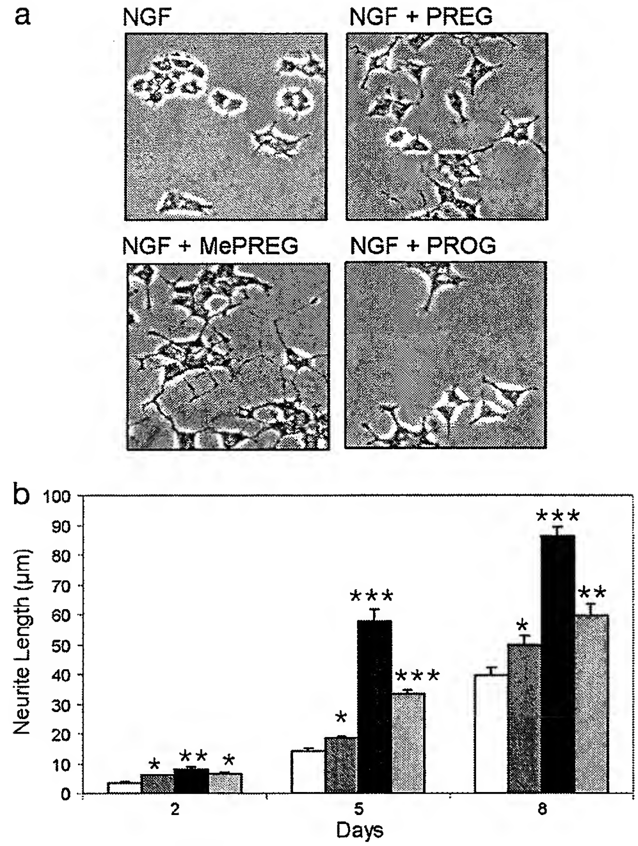


Fig. 2. PREG, MePREG, and PROG stimulate neurite outgrowth. (*a*) Steroid-mediated enhancement of neurite outgrowth in PC12 cells. Cells were treated with NGF (10 ng/ml) without or with PREG, MePREG, or PROG (30 μM) for 6 days and immunostained with anti-α-tubulin Ab. Neurite extension was markedly stimulated by all three steroids. (*b*) Time course of steroid-stimulated neurite outgrowth in PC12 cells. Cells were treated with NGF (white bar) (10 ng/ml) without or with PREG (dark gray bar), MePREG (black bar), or PROG (light gray bar) (30 μM) and were photographed after 2, 5, and 8 days. Neurite length was quantified from random photographs as described in *Materials and Methods*. Results represent means ± SEM for three independent observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. controls (Newman–Keuls test, ANOVA). MePREG was the most active.

The average neurite length of PC12 cells was measured. Without NGF treatment, neither PREG nor MePREG nor PROG induced neurite outgrowth, whereas every one of these steroids stimulated neurite outgrowth of cells treated with NGF concentrations in the range of 10–100 ng/ml (data not shown). After exposure of PC12 cells to NGF (10 ng/ml) for 5 days, the average neurite length was 14 μm. In the presence of 30 μM PREG, it reached 19 μm; in the presence of 30 μM PROG, it reached 33 μm; and in the presence of 30 μM MePREG, it reached 58 μm (Fig. 2*b*). Neurite extension was continued at 8 days.

Protection Against Microtubule Depolymerization Induced by Nocodazole. Nocodazole is a microtubule depolymerizing agent. There is one binding site for nocodazole per tubulin monomer, and nocodazole is bound specifically to the β-subunit (13), leading to disruption of microtubule assembly. Several concentrations of nocodazole were tested in the rat brain microtubule polymerization assay; maximal decrease of microtubule assembly (approximately half the control level) was achieved with 10 μM nocodazole (14). Then, microtubule assembly was monitored in the presence of both nocodazole and either 40 μM PREG or 40 μM MePREG (Fig. 3*a*). Nocodazole decreased the efficiency of both steroids to stimulate microtubule assembly. As a result, the amounts of microtubules formed remained slightly above the level that was achieved in the absence of both steroid and nocodazole. The effect of PROG was not investigated because it did not stimulate microtubule polymerization.

The effect of nocodazole was tested also in PC12 cells (Fig. 3*b*). These cells were cultured for 3 days in the presence of NGF (10 ng/ml) to induce neurite outgrowth. Then, they were incubated without steroid (control cultures) or with 20 μM PREG or MePREG for 1 h (a short time of exposure to steroids that had no effect on neurite length). Then, 30 μM nocodazole was added for 15 min. In control cultures, nocodazole induced a prominent retraction of neurites from an average length of 10.6 μm to an average length of 6.75 μm. However, nocodazole was ineffective on PREG- or MePREG-pretreated cells.

MAP2 Immunostaining. PC12 cells were kept in culture in the presence of NGF for 3 or 6 days without or with PREG, MePREG, or PROG. The MAP2–152 mAb was used. MAP2 is both soluble and bound to microtubules. In the conditions that were used, only the fraction of MAP2 that bound to microtubules was detectable by immunocytochemistry.

The intensity of MAP2 immunostaining in PC12 cells, grown in presence of NGF (10 ng/ml) for 3 days, was increased both in

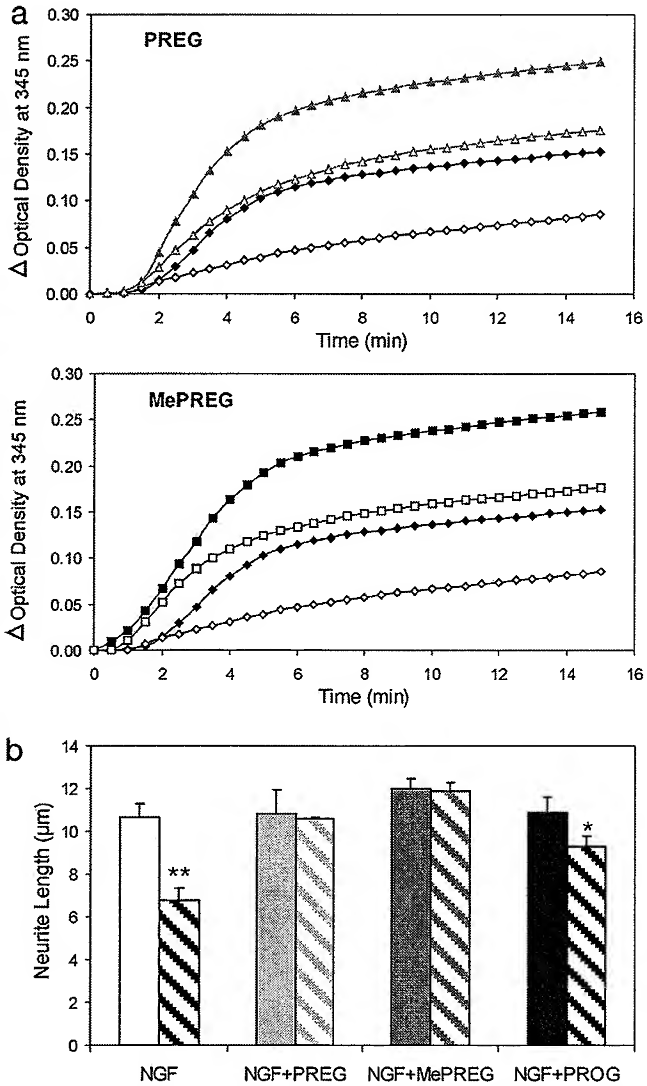


Fig. 3. Steroids counteract nocodazole effects. (*a*) Inhibition of microtubule polymerization by nocodazole and partial protection by either PREG or MePREG. Rat microtubule assembly was monitored at 345 nm at 37°C and recorded every 30 s for 15 min without (filled symbols) or with (open symbols) 10 μM nocodazole, and without steroid (black diamonds) or with 40 μM PREG (gray triangles) or 40 μM MePREG (black squares). Results represent means for four independent observations. (*b*) Neuroprotection. Shortening of neurites by nocodazole and its prevention by steroids is shown. PC12 cells were grown in NGF (10 ng/ml) containing media for 3 days and then incubated with PREG, MePREG, or PROG (20 μM) for 1 h, followed by incubation with either DMSO (filled bars) or 30 μM nocodazole (hatched bars) for 15 min. Neurite length was quantified from random photographs as described in *Materials and Methods*. Results represent means ± SEM for three independent observations. *, $P < 0.05$; **, $P < 0.01$ vs. control (Newman–Keuls test, ANOVA). PREG and MePREG prevented neurite shortening; PROG was less effective.

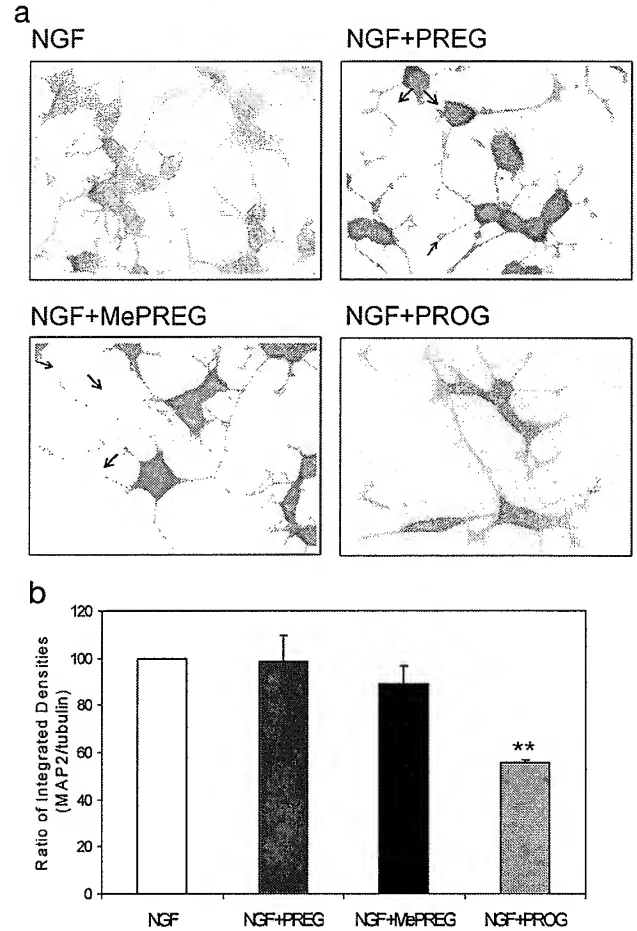


Fig. 4. Immunostaining of MAP2 and of tubulin. (*a*) MAP2 immunostaining in PC12 cells. Cells were cultured for 3 days with 10 ng/ml NGF without or with PREG, MePREG, or PROG (20 μM). The anti-MAP2 152 mAb was used. PREG and MePREG increased the brown immunostaining of both cell bodies and neurites (arrows). However, immunostaining after PROG exposure was the same as in cells treated by NGF alone. (*b*) Densitometric analysis of MAP2 and α-tubulin Western blotting of PC12 cell extracts. Ratios of MAP2/tubulin integrated densities were set at 100% for control cultures. Results represent means ± SEM for four independent observations. **, $P < 0.01$ vs. control (Newman–Keuls test, ANOVA). There were no significant differences between PREG or MePREG-treated cells and control cultures, whereas the MAP2/tubulin ratio was decreased by PROG treatment.

cell bodies and in neurites after exposure to 20 μM PREG or MePREG, but not to 20 μM PROG (Fig. 4*a*).

In three independent experiments, Western blot analysis showed that, in the conditions that were used (60 μg of protein loaded on each lane), the relative amounts of tubulin and of MAP2, extracted from PC12 cells treated with NGF (10 ng/ml) for 6 days, did not change in the presence of 30 μM PREG or MePREG, as shown by the ratios of MAP2 to tubulin integrated densities, whereas 30 μM PROG led to a decrease of the MAP2/tubulin ratio (Fig. 4*b*). This observation does not indicate that PROG selectively stimulates tubulin synthesis because the amounts of tubulin were not increased in Western blot analysis. It is more likely that PREG and MePREG increased the fraction of MAP2 linked to tubulin, thus protecting it from proteolysis. Such protection did not occur with PROG, therefore leading to a decrease of MAP2 amount.

PC12 Neurite Outgrowth After MAP2 Suppression by RNAi. To provide more conclusive evidence for the role of MAP2 in steroid regulated neuritogenesis, the RNAi technology was used. Two 21-bp-long small interfering RNA (siRNA) duplexes specific of MAP2 transcripts were selected and sequentially used. The effectiveness of siRNA was shown by the decrease of MAP2 protein in NGF-treated cells, observed by immunoblotting analysis performed with the mAb AP20 by using Hsp70 as reference standard (Fig. 5*a*). The prominent decrease of MAP2 expression was accompanied by a complete loss of the stimulatory effects of PREG and MePREG on neurite extension, contrasting with

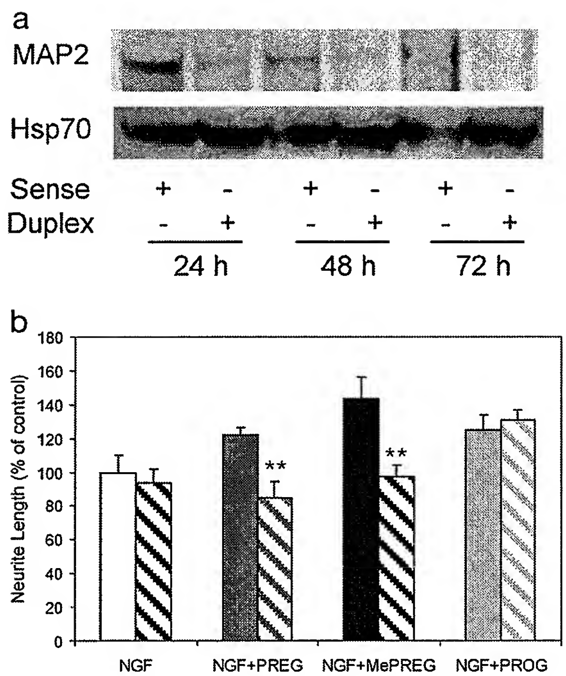


Fig. 5. Inhibition of MAP2 expression by 21-nt siRNA in PC12 cells. Suppression of PREG/MePREG enhancement of neurite outgrowth is shown. (*a*) Immunoblotting of MAP2 (AP20 Ab) and of Hsp70: PC12 cells were cultured in the presence of NGF (50 ng/ml) for 11 days and then transfected with MAP2–siRNA duplex or sense oligonucleotide for 24–72 h. We loaded 60 μg of protein in each lane. (*b*) PC12 Cells were transfected by MAP2-siRNA sense single strand taken as control (filled bars) or by MAP2-siRNA duplex (hatched bars) for 6 h and treated with NGF (50 ng/ml) and either PREG or MePREG or PROG (30 μM) for 48 h. Results represent means ± SEM for three independent observations. **, $P < 0.01$ vs. sense control (Newman–Keuls test, ANOVA). The enhancement of neurite outgrowth by PREG and MePREG was completely suppressed by the MAP2–siRNA, whereas the effect of PROG was unchanged.

an unchanged effect of PROG (Fig. 5*b*). Identical results were obtained with both MAP2–siRNAs. Experiments were also made with two times greater amounts of siRNA duplexes, which produced a major decrease of NGF-induced neurite extension (data not shown).

Discussion

The specific suppression of MAP2 synthesis with antisense oligonucleotides was shown to inhibit minor neurite formation in cultured cerebellar macroneurons (15) and the development of neuronal polarity in cultured hippocampal neurons (16), thus confirming that neuronal MAPs have an important role in neuronal morphogenesis. They promote the assembly and increase the stability of microtubules and form cross-bridge structures between microtubules (bundling) as well as between microtubules and other cytoskeletal elements, thus being involved in the extension of the dendritic tree. Conversely, the outgrowth of cytoplasmic elongations similar to neurites was observed when MAP2 was overexpressed in cultured nonneuronal cells (17).

The PC12 line is a convenient alternative to cultured neurons because it elongates neuritic processes when cultured in the presence of NGF, and the resulting extensions are filled with microtubules (18). MAP2 becomes detectable in PC12 cells after 4 days of exposure to NGF; then, its amount increases 5- to 7-fold when exposure to NGF lasts 12 days, concomitant with intense neurite growth.

The MAP2 family of MAPs is the only demonstrated target of the neurosteroid PREG, as shown by microtubule binding and microtubule polymerization experiments *in vitro* (6). The active concentration of PREG, in the range of 10 μm, may be of physiological significance in the context of neurosteroids synthesized in CNS and endowed with paracrine and/or autocrine activities (3, 5). The location of the PREG-binding site on MAP2 isoforms is not known, although preliminary experiments performed with recombinant MAP2C have indicated that this short isoform also binds PREG (E. Plassart-Schiess, V.F.-L., and E.-E.B., unpublished work). Another steroid, dehydroepiandrosterone, has been found to bind recombinant MAP2C (19), although no function was assigned to this binding. However, MAP2 purified from rat brain poorly binds dehydroepiandrosterone, a likely consequence of posttranslational modifications (6).

Here, we show that the microtubules prepared from the extracts of PC12 cells behave as rat brain microtubules as regards the stimulation of polymerization by PREG and MePREG. This effect *in vitro* is paralleled by the stimulation of neurite extension induced by both steroids in NGF-treated PC12 cells. An additional argument in favor of PC12 microtubules as the target of PREG and MePREG is the protective effect of both steroids against the microtubule retraction induced by nocodazole. However, MAP2 is not the only microtubule associated protein involved in the extension of neurites by PC12 cells. Tau is also involved in the stabilization of microtubules and consequently in neurite morphology (17). Tau antisense oligonucleotides decrease neurite extension of PC12 cells, whereas Tau overexpression renders neurites resistant to nocodazole (20). Attenuation of MAP1B expression by antisense oligonucleotides also inhibits initiation of neurite outgrowth (21).

Therefore, a direct demonstration of MAP2 involvement in the stimulation of neurite extension of PC12 cells was mandatory. The RNAi approach permitted such a demonstration when we used short siRNA duplexes to transfect PC12 cells that were treated with NGF or left untreated, as already shown with neurons (22). After 11 days of culture in presence of NGF (50 ng/ml), sufficient expression of MAP2 was achieved, permitting clear demonstration of high-molecular-weight MAP2 by Western blotting. Potent inhibition of MAP2 expression by transfection of siRNA duplexes occurred. The measurement of neurite extension was performed after only 2 days of culture, a convenient time interval in which to observe the stimulatory effects of steroids. At that time, the average length of neurites was almost unchanged by transfection of both siRNA duplexes, a likely consequence of persisting low levels of MAP2 expression or of compensatory roles of other MAPs (MAP1B and Tau), but the stimulatory effects of PREG and MePREG on neurite extension were completely lost. Therefore, we concluded that the stimulatory activity of both steroids on MAP2-induced microtubule polymerization is involved in the stimulation of neurite extension by PC12 cells, defining an intracellular signaling system.

PROG was reported to reduce apoptosis of PC12 cells after serum and NGF withdrawal (23). The effect of PROG was blocked by a PROG nuclear receptor antagonist. Stimulation of neurite extension by PROG, as described here, did not seem to be mediated by the PROG receptor because the active concentrations were much larger than those that were needed to activate a nuclear receptor. However, other types of PROG receptors have been described (24). Such a PROG effect did not seem to involve MAP2 because the siRNA-induced decrease of MAP2 expression did not impede the stimulatory activity of PROG.

In conclusion, PREG and its synthetic analog MePREG stimulate microtubule polymerization from rat brain and from PC12 cells. In pheochromocytoma cells that are exposed to NGF, both steroids stimulate the extension of neurites. RNAi experiments demonstrate that the inhibition of MAP2 expression results in the suppression of this stimulatory effect. This

work describes a mechanism of neurosteroid action and shows that MAP2 is the target of PREG in this effect.

Materials and Methods

Animals. Male adult Sprague–Dawley rats (body weight, 300 g) were obtained from Janvier (Le Genest-St-Isle, France). Animal care was in accordance with the European Communities Council Directive of November 24, 1986. Animals were killed by decapitation, and their entire brains were used immediately for the preparation of microtubules.

Steroids. PREG and PROG were purchased from Sigma, and MePREG was either obtained from Steraloids (Newport, RI) or synthesized and guaranteed to be >97% pure by Roowin (Romainville, France)

Abs. Anti-α-tubulin mAb SC 5286 was purchased from Santa Cruz Biotechnology. mAb 152 (25) was shown to react specifically with high-molecular-weight MAP2. The anti-Hsp70 mAb was purchased from StressGen Biotechnologies (Victoria, Canada), and anti-MAP2 mAb AP20 was purchased from Sigma.

Preparation of Microtubules and Assay of Microtubule Assembly. Microtubules were prepared from the brains of 50 adult rats by a temperature-dependent *in vitro* assembly–disassembly procedure (26). Briefly, rat brains were homogenized in a volume of buffer A (0.1 M Mes/1 mM EGTA/0.1 mM EDTA/1 mM $MgCl_2$/1 mM DTT/1 mM PMSF/1 mM GTP, pH 6.4) in a volume (expressed in milliliters) equal to the weight of the pool of brains (expressed in grams), for three 30-s pulses separated by 30-s intervals. After centrifugation of homogenate at 105,000 $\times$ g and 4°C for 1 h, 2 M glycerol was added to the supernatant, and the mixture was incubated at 37°C for 30 min. The polymerized preparation obtained was deposited on a cushion of buffer A, supplemented with 1.5 M glycerol. After centrifugation at 105,000 $\times$ g and 20°C for 2 h, the final pellets were rinsed with buffer A and stored at −80°C for later use. When required, microtubule pellets were unfrozen and incubated in buffer A at 4°C for 1 h. The solutions of depolymerized microtubules were clarified by centrifugation at 30,000 $\times$ g at 4°C for 35 min. Protein concentrations were determined by BC assay (Uptima Interchim, Montluçon, France). The microtubule proteins (1 mg of protein per ml) were incubated with either steroid at concentrations of 40 μM (final ethanol concentration, 1.3%) without or with nocodazole (Sigma) at a concentration of 10 μM (final DMSO concentration, 1%). Control incubations contained the same concentration(s) of solvent(s). Microtubule assembly was monitored by the increase of absorbance at 345 nm vs. time, with an Uvicon spectrophotometer (Kontron, Montigny-le-Bretonneux, France), equipped with an automatic six-sample changer thermostated at 37°C for either 15 or 30 min.

Culture of PC12 Cells. Cells were plated on plastic dishes coated with poly(L-lysine) and maintained in DMEM supplemented with 10% horse serum and 5% FBS, in an incubator set at 37°C and 5% CO_2. Cell culture was performed on 12-well plates (2 $\times$ 10^4 cells per well) for immunocytochemistry and quantification of neurite outgrowth and on six-well plates (3 $\times$ 10^5 cells per well) or 100-mm Petri dishes (1 $\times$ 10^6 cells per dish) for transfection assay and immunoblotting. After ≈6 h, the differentiated neuronal phenotype was induced by adding NGF (10 ng/ml), with or without test agent(s) in fresh DMEM supplemented with 2% horse serum and 1% FBS. All cultures were fed every 2 or 3 days with fresh medium. The steroids were added at concentrations of either 20 or 30 μM (the final concentration of ethanol was 0.1%). To study steroid effects on neurite retraction, cells were treated with NGF for 3 days and then incubated with steroids for 1 h, followed by 30 μM nocodazole for 15 min (dissolved in DMSO at a final concentration of 0.1%). Control cultures contained the same concentration(s) of solvent(s). At different time points during the treatment, cells were either photographed or fixed with 4% paraformaldehyde for later examination.

siRNA Preparation and Transfections. siRNAs corresponding to MAP2 mRNAs were designed as recommended in ref. 27, with 5′ phosphate, 3′ hydroxyl, and 2-bp overhangs on each strand; they were synthesized by Dharmacon (MWG, Courtaboeuf, France). The following gene-specific sequences were used according to Krichevsky and Kosik (22): siRNA1, 5′-CAGGGC-ACCUAUUCAGAUAdTdT-3′ (sense) and 5′-UAUCUGAA-UAGGUGCCCUGdTdT-3′ (antisense). The following second set of gene-specific sequences was designed according to the recommendations of Tuschl *et al.* (27): siRNA2, 5′-UUCGCU-GAGCCUUUAGACAdTdT-3′ (sense) and 5′-UGUCUAAA-GGCUCAGCGAAdTdT-3′ (antisense). Annealing for duplex siRNA formation was performed according to the manufacturer's instructions.

Cells were maintained in DMEM supplemented with 10% horse serum and 5% FBS. Transfection experiments were initiated 3 or 4 days after plating. Lipofectamine (Invitrogen) diluted in DMEM was applied to the 21-nt duplexes (or to sense oligonucleotide used as control) diluted in OptiMEM, and the formulation was continued for 30 min. Transfections were performed in six-well plates coated with poly(L-lysine) for quantification of neurite outgrowth (3 $\times$ 10^5 cells per well, 7 μl of Lipofectamine, and 120 pmol of 21-bp duplex or sense RNA and DMEM to a final volume of 1 ml per well) and on 100-mm Petri dishes coated with poly(L-lysine) for immunoblot analyses (1.5 $\times$ 10^6 cells per dish, 40 μl of Lipofectamine, and 315 pmol of 21-bp duplex or sense RNA and DMEM to a final volume of 2.5 ml) for ≈6 h. For quantification of neurite outgrowth, 1 ml of DMEM was added to each well, the transfected cells were resuspended, and 0.5 ml of each suspension was seeded in 12-well plates, coated with poly(L-lysine) and containing 0.5 ml of DMEM supplemented with 20% horse serum, 10% FBS, and NGF (final concentration, 50 ng/ml). Steroids (30 μM) were added when indicated. Cells were photographed 48 h after the addition of steroid. For immunoblot analyses, 2.5 ml of DMEM supplemented with 20% horse serum, 10% FBS, and NGF (final concentration, 50 ng/ml) were added to each dish. Proteins were extracted at 24–72 h after transfection.

Measurement of Neurite Outgrowth. Random field photographs of NGF-treated PC12 cells were analyzed with IMAGE (Scion, Frederick, MD). Average neurite length (mean ± SEM) was determined by measuring the longest neurite of at least 200 cells randomly selected in each of three wells.

Immunocytochemistry. After two washes with PBS for 5 min, the cells were fixed with PFA on the bottom of Petri dishes, then incubated in PBS containing 3% BSA and 0.1% Triton X-100 at room temperature for 45 min. Cells were washed again then incubated at 4°C overnight in the presence of either anti-α-tubulin mAb (1:1,000 dilution) or anti-MAP2 mAb 152 (1:5,000 dilution). After two washes with PBS, rabbit anti-mouse Ig biotin-conjugated IgG, F(ab′)$_2$ fragment (1:500 dilution; Roche Diagnostics) was added at room temperature for 45 min. After three washes, the streptavidin–peroxydase amplification complex (Vectastain kit; AbCys, Paris, France) was added at room temperature for 45 min. The cells were then washed, and the aminoethyl carbazole substrate (Sigma) was deposited on the cells for 15 min. The reaction was stopped with distilled water, and the cells were counterstained with hematoxylin and mounted in Glycergel (DAKO, Serotec).

Total PC12 Cell Extract and Cytosol Enriched in Microtubule Proteins. PC12 cells (cultured to confluence on 100-mm dishes) were rinsed, removed by gentle trituration in PBS, and centrifuged at 500 × *g* for 4 min, and the cell pellets were kept at −80°C. For electrophoreses and immunoblot analyses, the frozen pellets were boiled for 5 min in sample buffer, containing 2% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris·HCl (pH 6.8), and 10% glycerol (total cell extracts). To assay microtubule polymerization, the pellets were homogenized in a Dounce glass homogenizer with buffer A containing protease inhibitor mixture Complete (Roche Diagnostics), left at 4°C for 1 h to induce microtubule depolymerization, and centrifuged at 100,000 × *g* at 4°C for 45 min. The supernatants that were enriched in microtubule proteins (4 mg/ml) were assayed immediately or stored at −80°C until analysis. Protein concentrations were determined with the BC assay.

Western Blot Analysis. Total cell extracts (60 μg of protein) were separated on 10% SDS/PAGE gels or on 3–8% Tris·acetate gels (NOVEX, San Diego; Invitrogen) and then transferred to nitrocellulose membranes. The membranes were blocked by incubation in 5% skim milk in TBS buffer (0.1% Tween 20/10 mM Tris/50 mM NaCl, pH 8.8) at room temperature for 1 h. All washes were performed in TBS buffer. The membranes were then incubated at 4°C overnight with all of the following mAbs (dilutions given in parentheses): anti-tubulin (1:500), anti-MAP2 152 (1:1,000), anti-MAP2 AP20 (1:200), and anti-Hsp70 (1:1,000). Incubation with the secondary goat anti-mouse Ig peroxydase-conjugated IgG $F(ab')_2$ fragment (1:10,000) (Perbio Science, Brebières, France) was performed in TBS buffer containing 5% skim milk for 45 min at room temperature. The signal was detected by the enhanced chemiluminescence system (Amersham Pharmacia), according to the manufacturer's instructions, with Kodak X-Omat film and analyzed for quantification with IMAGE.

Statistical Analyses. One-way ANOVA was applied to determine significant differences between control and treated conditions. The level of significance was set at $P < 0.05$. The Newman–Keuls post hoc test was used.

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